Chemosphere 220 (2019) 556–564

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

The role of humic acids on gemfibrozil toxicity to zebrafish embryos

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HIGHLIGHTS

- Humic acids (HA) increased gemfibrozil LC_{50} to zebrafish embryos.
- The HA protective role observed at lethal level was not verified at sublethal levels.
- HA *per se* affected biochemical, behavioural and genotoxic parameters.
- Testing endpoints at different levels of biological organization is recommended.
- Dissolved organic carbon should be considered when assessing the toxicity of pharmaceuticals.

ARTICLE INFO

Article history:
Received 16 October 2018
Received in revised form
17 December 2018
Accepted 18 December 2018
Available online 19 December 2018

Handling Editor: David Volz

Keywords:
Pharmaceuticals
Fibrates
Comet assay
Locomotor behaviour
Biomarkers

ABSTRACT

Climate change is expected to alter the dynamics of water masses, with consequent changes in water quality parameters such as dissolved organic carbon (DOC) concentration. DOC levels play a critical role in the fate of organic chemicals, influencing their bioavailability and toxicity to aquatic organisms. This study aimed to evaluate the effects of DOC, particularly humic acids (HA), in the toxicity of gemfibrozil (GEM) - a human pharmaceutical frequently detected in surface waters. Lethal and sublethal effects (genotoxic, biochemical and behavioural alterations) were evaluated in zebrafish embryos exposed to several concentrations of GEM and three HA levels, in a full factorial design. HA significantly increased GEM LC_{50} values, mainly in the first 72 h of exposure, showing a protective effect. At sublethal levels, however, such protection was not observed since HA *per se* elicited adverse effects. At a biochemical level, individual exposure to HA (20 mg/L) elicited significant decreases in cholinesterase and glutathione *S*-transferase activities. Regarding behaviour, effects of individual exposure to HA appear to surpass the GEM effects, reducing the total distance moved by larvae. Both GEM and HA significantly increased DNA damage. Hence, this study demonstrated that abiotic factors, namely HA, should be considered in the assessment of pharmaceuticals toxicity. Moreover, it showed that lethality may not be enough to characterize combined effects since different patterns of response may occur at different levels of biological organization. Testing sublethal relevant endpoints is thus recommended to achieve a robust risk assessment in realistic scenarios.

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1. Introduction

The effects of climate change on temperature and precipitation may lead to an increased frequency of both floods and droughts, with high impact on the water quality parameters of aquatic systems. Dissolved organic carbon (DOC) is a ubiquitous component of surface waters that derives from the incomplete decomposition of organic material. Despite the generally low DOC concentrations in freshwaters (below 4 mg C/L), values higher than 50 mg C/L can be found (Laudon et al., 2012; Matsuo et al., 2006). DOC is mainly composed by humic substances, including humic, fulvic and

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https://doi.org/10.1016/j.chemosphere.2018.12.133
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hydrophilic acids, varying in its composition and abundance (Ma et al., 2001). DOC is of major importance to aquatic biota, representing an energy substrate for some aquatic organisms and a modulator of the aquatic food web structure (Haitzer et al., 1998; Hessen and Anderson, 2008). It also plays a critical role as a transport vector in the dispersion of metals and organic chemicals, influencing their bioavailability and toxicity (Haitzer et al., 1998; Matsuo et al., 2004). On the other hand, DOC can also cause stress response reactions (see review by Steinberg et al. (2006)), such as effects on sodium uptake kinetics and loss rates (Matsuo et al., 2004). Among DOC, special attention has been given to humic acids (HA) due to their higher affinity for binding organic chemicals especially hydrophobic compounds (De Paolis and Kukkonen, 1997).

Increasing DOC concentrations and fluxes, mainly attributed to increased temperature and precipitation (Laudon et al., 2012; Worrall et al., 2004), have been observed in surface waters of the northern hemisphere. This trend raises concerns due to the possible effects on aquatic biota, including DOC influence on the toxicity of chemicals. Previous studies reported that increased DOC concentration reduced the bioavailability and toxicity of metals (Al-Reasi et al., 2011) and the toxicity and bioconcentration of organic chemicals (De Paolis and Kukkonen, 1997; Haitzer et al., 1998; Kadlec and Benson, 1995) in aquatic biota. However, there is scarce knowledge about DOC influence on the toxicity of emerging pollutants such as pharmaceuticals.

Pharmaceuticals are chemicals designed to have a specific biological activity, being widely used in human and veterinary medicine. Increased consumption of pharmaceutical products combined with the inefficiency of wastewater treatment plants to completely remove these compounds from wastewater, leads to increasingly higher concentrations in surface waters (Richardson and Ternes, 2014) with potentially pernicious effects on non-target organisms. Fibrates, such as gemfibrozil (GEM), are one of the classes of pharmaceuticals most frequently detected in water (Santos et al., 2010). GEM has been found in surface waters across Europe and the United States (Loos et al., 2009; Schwab et al., 2005), at concentrations as high as 1.55 mg/L (Schwab et al., 2005). As a lipid regulator, GEM is used to reduce plasma triglycerides and total cholesterol, and to increase high-density lipoproteins (Araujo et al., 2011). Several effects of GEM have been reported on aquatic organisms, particularly fish. For instance, alteration of lipoproteins composition was observed in rainbow trout (*Oncorhynchus mykiss*), which may result in a reduction of the nutritional quality of the organisms (Prindiville et al., 2011); potential endocrine disruption was observed in goldfish (*Carassius auratus*) (Mimeault et al., 2005); yolk malabsorption syndrome was found in zebrafish (*Danio rerio*) embryos, leading to insufficient yolk sac reabsorption, growth delay and morphogenic defects (Raldúa et al., 2008) and genotoxic effects were found in *Sparus aurata* (Barreto et al., 2017). Furthermore, swimming behaviour disruption was observed in zebrafish larvae exposed to GEM concentrations higher than 3 mg/L (Henriques et al., 2016). These effects, allied to the slow degradation of GEM in surface waters (Grenni et al., 2013), highlight the need to assess its effects in aquatic systems, under relevant exposure scenarios.

Considering that most of the available studies assessing the toxicity of GEM to aquatic biota do not take into consideration the influence of water quality parameters, this study aimed to assess the effect of HA (10 and 20 mg/L) on GEM toxicity (from 0.0016 up to 33.3 mg/L), using zebrafish embryos as model organisms. We hypothesized that increasing HA concentrations, would decrease GEM toxicity to zebrafish. Effects were evaluated at several levels: biochemical, cellular and behavioural. Biochemical markers have been widely used as early stressor detectors due to their high sensitivity. A previous study found significant effects of GEM in several biomarkers in the marine fish gilthead seabream (*Sparus aurata*) (Barreto et al., 2018). However, there is scarce knowledge on the effects of this pharmaceutical on enzymatic activities of zebrafish. For this reason, a battery of enzymatic biomarkers was selected in this study: cholinesterase (ChE), glutathione-*S*-transferase (GST), catalase (CAT) and lactate dehydrogenase (LDH). ChE is involved in normal nerve function, playing a determinant role in several functions, such as prey location and evasion (Bretaud et al., 2000; dos Santos Miron et al., 2005). The inhibition of this enzyme leads to accumulation of acetylcholine in synapses resulting in nervous system function disruption, that may cause irregular neuronal and muscular development (Behra et al., 2002). Both GST and CAT are enzymes involved in mechanisms of oxidative stress prevention. GST catalyses the conjugation of glutathione with xenobiotic compounds, enabling their excretion whereas CAT fights oxidative stress by decomposing hydrogen peroxide into oxygen and water. On the other hand, LDH is an enzyme with a key role in the anaerobic pathway of energy production, converting pyruvate into lactic acid through glucose. Effects at the cellular level were assessed by studying the genotoxic potential (DNA damage) through the comet assay. This is a highly relevant endpoint considering that DNA integrity loss can lead to mutations, chromosome aberrations, cancer and other irreversible toxic effects (Oliveira et al., 2010, 2007; Rocco et al., 2012). The comet assay has been recognized as a sensitive method to detect primary DNA damage in various fish species caused by different contaminants (Barreto et al., 2017; Hseu et al., 2008).

Zebrafish behavioural assays have been increasingly used as a powerful tool to assess the toxicity of chemicals mainly due to its sensitivity and suitability for large scale screening (MacPhail et al., 2009; Padilla et al., 2011). In the present study, the behavioural effects of GEM were assessed by determining the total swimming distance as a measure of swimming performance.

2. Materials and methods

2.1. Test organisms

Zebrafish (*D. rerio*) wild type embryos were used as a biological model and supplied by the laboratory culture kept at the Department of Biology (University of Aveiro, Portugal). Organisms kept in a recirculating system (ZebTEC; Tecniplast) with reverse osmosis and activated carbon filtered tap water, complemented with salt "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA) (automatically adjusted for pH and conductivity) were used as a source of embryos for the experimental assays. Animals are maintained in aquaria with water temperature of $26.0 \pm 1\,^{\circ}C$, conductivity $750 \pm 50\,\mu S/cm$, pH 7.5 ± 0.5, salinity of 0.35 ppt and dissolved oxygen equal or above 95% saturation at a 16:8 h (light: dark) photoperiod cycle. The adult fish are fed daily with commercial artificial diet (ZM-400 fish food; Zebrafish Management Ltd).

Zebrafish eggs, used for this study, were collected within 30 min after natural mating, rinsed in fish culture water, and screened using a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation) to exclude eggs unfertilized, injured or with cleavage irregularities.

2.2. Test chemicals

GEM (5-(2,5-dimethylphenoxy)-2,2-dimethyl-pentanoic acid) was acquired from Tokyo Chemical Industry (Japan), HA (ash ~20%) and an isotope-labelled internal standard (IS) of diclofenac ($^{13}C_6$) were purchased from Sigma-Aldrich®. All other chemicals were of analytical grade, obtained from Sigma–Aldrich, Bio-Rad and Merck.

Liquid chromatography–mass spectrometry (LC–MS) grade methanol and acetonitrile (Li Chrosolv Hypergrade) were purchased from Merck (Darmstadt, Germany). Formic acid for the mobile phase's acidification was purchased from Labicom (Olomouc, Czech Republic). Ultra-pure water was produced using an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea). All compounds used were analytical standards or of high purity (>98%).

2.3. Determination of GEM in water using liquid chromatography-tandem mass spectrometry

The chemical analyses of GEM in the exposure solutions samples (section 2.5) were performed using a TSQ Quantiva triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela 1250 LC pump (Thermo Fisher Scientific) and an HTS XTCTC autosampler (CTC Analytics AG, Zwingen, Switzerland). For the analytical procedures, stock solutions of GEM and IS were prepared in methanol at a concentration of 1 mg/mL and stored at −20 °C. A spiking mixture was prepared, for each compound, by diluting stocks in methanol to a final concentration of 1 μg/mL, and stored at −20 °C. Thawed water samples were filtered through a syringe filter (0.45 μm, regenerated cellulose, Labicom, Olomouc, Czech Republic), after that 10 ng of IS was added to each sample. An analytical Hypersil GOLD aQ column (50 mm length, 2.1 mm i.d, 5-μm particles; Thermo Fisher Scientific) was used as analytical column for chromatographic separation of target compounds. Heated electrospray ionization (HESI) was used to ionize analytes. Method parameters are reported in Tables S1 and S2 (Supplementary Material). This method was validated in the range of tested concentrations and exhibited good linearity ($R^2 = 0.997$) in the concentration range between 1 and 50 mg/L.

The limit of quantification (LOQ) for simultaneous analysis of GEM was determined by measuring aqueous standard solutions in a concentration range from 1 to 50 mg/L. LOQ was calculated as one quarter of the lowest calibration point response in the calibration curve, where relative standard deviation of average response factor was <30%. Peak area corresponding to this concentration was used to calculate LOQ for GEM in each sample. Average LOQ was 1.04 mg/L. Matrix-matched standard response was used as factors for correcting the response derived from the calibration curve. Matrix matched standard was prepared from tested water blank by spiking with both IS and native compound at a concentration of 25 mg/L.

2.4. Exposure conditions

Zebrafish eggs were exposed to several concentrations of GEM and three concentrations of HA, following a full factorial design (Table 1). GEM stock solutions were prepared by dissolution in ethanol and filling up with fish culture water (water from the recirculating system). The tested concentrations were obtained by dilution of the GEM stock solution in fish culture water. HA levels were obtained by dissolving HA in fish culture water (GEM = 0 mg/L) or directly in the GEM solutions. The ethanol concentration in the tested solutions did not exceed 0.002% (v/v), which was previously reported to have no effects on zebrafish mortality (Reimers et al., 2006). In a preliminary assay, the highest ethanol concentration used in this study induced no significant effects on the locomotor of zebrafish (*t*-test, $p > 0.05$).

Four different sets of experiments were performed in this study: 1) Fish embryo toxicity test (FET); 2) Biochemical markers assay; 3) Behaviour analysis and 4) Genotoxicity (comet) assay. In all assays, the newly fertilized eggs (3 h post-fertilization- hpf) were kept in the dark, under controlled temperature (27 ± 1 °C) to avoid GEM photodegradation.

Table 1
Concentrations used to test the effects of the levels of humic acids (HA) on gemfibrozil (GEM) toxicity to zebrafish embryos in the different bioassays. A full factorial design was used in each bioassay (combining the GEM concentrations with all the HA levels).

	GEM (mg/L)	HA (mg/L)
Fish Embryo Toxicity test (FET)	0	0
	6.6	5
	9.9	20
	14.8	
	22.2	
	33.3	
Biochemical test & Comet assay	0	0
	0.0016	10
	0.008	20
	0.04	
	0.2	
	1	
	5	
Behaviour assay	0	0
	0.04	10
	0.2	20
	1	
	5	

2.5. Fish embryo toxicity (FET) assay

The fish embryo toxicity test followed the OECD guideline 236 (OECD, 2013). GEM concentrations were chosen based on the work of Henriques et al. (2016). Embryos (10 embryos per replicate; 3 replicates per treatment) were placed individually in the wells of 24 well plates, with 2 mL of test solution, for 96 h. Plates were covered to avoid evaporation. Embryos were observed daily under a stereomicroscope and mortality recorded. Based on the results of this test, sublethal concentrations were selected for the following assays (biochemical, behaviour and genotoxicity). At the beginning of the assay, samples of the exposure solutions were collected for GEM quantification and kept frozen until analysis.

2.6. Biochemical assay

For the biochemical analysis, 10 embryos per replicate (5 replicates per treatment) were exposed, for 96 h, in Petri dishes. After exposure, larvae of each replicate were pooled together, snap-frozen in microtubes with 800 μL of phosphate buffered saline solution (PBS, 0.1 mM, pH 7.4) and stored at −80 °C until further processing. On the day of the analyses, samples were slowly defrosted in ice, homogenized using a sonicator (Branson S-250A) and centrifuged at 4 °C, 10,000 *g*, during 20 min, to isolate the post-mitochondrial supernatant. Enzymatic activities were determined spectrophotometrically (Thermo Scientific Multiskan® Spectrum), in quadruplicate, using 96 wells microplate and expressed in nanomoles of substrate hydrolyzed per minute per mg of protein. ChE activity was determined according to the method of Ellman (Ellman et al., 1961), using acetylthiocholine as substrate. The absorbance increase caused by the conjugation product of thiocoline (product of acetylthiocholine degradation) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) in PBS was measured at 414 nm for 5 min. GST activity was determined at 340 nm, as described by Habig and Jakoby (1981), by monitoring, during 5 min, the increase in absorbance resulting from the conjugation product between reduced glutathione and 1-chloro-2,4-dinitrobenzene. CAT activity was measured at 240 nm as described by Claiborne (1985) by monitoring the decrease of absorbance due to hydrogen peroxide degradation, during 2 min. LDH activity was measured at 340 nm, following the Vassault method (Vassault, 1983), by monitoring, for 5 min, the decrease in absorbance due to the oxidation of NADH.

Protein quantification was performed in quadriplicate using ɣ-globulin as standard, at 595 nm, according to the Bradford method (Bradford, 1976).

2.7. Behaviour assay

For the behaviour assay, 12 organisms per treatment were placed individually in 96-well plates, using a random design for the distribution of the treatments in the plate, to avoid bias. Only larvae without visible malformations were used for this assay. Locomotion was evaluated at 120 hpf to ensure that all larvae had their swimming bladder inflated and, therefore, able to swim freely. At this stage of development, larvae still feed on their yolk sac. Locomotor activity was measured using a ZebraBox - ZEB 478 (Viewpoint Life sciences, Lyon, France). This system monitors movement by automated video recording using a tracking setting during periods of light/dark. After 5 min of acclimation in light, movement of each larva was recorded for 10 min in the dark followed by 5 min in the light, to assess effects of the chemical in the response to the stimulus (stillness and movement of larvae in light and dark conditions respectively). For background correction, a threshold of 30 was set. The total distance moved (TD, overall distance moved during a defined time period, in mm) was recorded.

2.8. Comet assay

For the comet assay, 15 embryos per replicate (3 replicates per treatment) were exposed in Petri dishes for 120 hpf. After the exposure period, embryos were killed by immersion in a saturated benzocaine (ethyl-4-aminobenzoate) solution. To perform cell isolation, a mechanical approach was used, generally following the procedures described by Kosmehl et al. (2008). Briefly, animals were gently mashed with a polypropylene pestle, in 1.5 mL microtubes containing PBS. The cell suspension was resuspended, filtered through a 70 μm cell strainer to another microtube and centrifuged for 10 min at 200 g (4 °C). Pellets were resuspended in 1 mL of PBS and centrifuged 7 min at 180 g (4 °C). The final pellet was resuspended in 150 μL of PBS and immediately used for cell viability (trypan blue exclusion test) and comet assay.

Only cell suspensions with cell viability higher than 80% were used for comet assay. The alkaline comet assay was conducted generally following the method of Singh et al. (1988) as described by Barreto et al. (2017). To control the electrophoresis conditions, negative (suspension of cells of control organisms) and positive (suspensions of cells treated with 25 μM of H_2O_2 for 10 min) controls were included for each electrophoresis run. Slides were coded to assure an unbiased scoring and analysed by scoring 100 randomly selected cells from each slide, according to tail length. Nucleus were ranked in 5 classes (Collins, 2004): class 0 - undamaged, without tail; class 1 - with tail shorter than the diameter of the head (nucleus); class 2 - with tail length 1–2 times the diameter of the head; class 3 - with tail longer than twice the diameter of the head; class 4 - with no heads. A damage index (DI), expressed in arbitrary units was calculated, according to the damage classes, using the formula:

$$DI = (0 \times n0) + (1 \times n1) + (2 \times n2) + (3 \times n3) + (4 \times n4)$$

where:

n_i (i = 0 to 4): number of nucleus ranked in class i.

2.9. Data analysis

The software R (Core Team, 2014) through the package drc, was used to calculate lethal concentration (LC_x) and slope values, by fitting dose-response curves. The LC_{50} values were compared by a generalized likelihood ratio test. A two-way analysis of variance (ANOVA) was performed on SigmaPlot software V12.5 (SysStat, San Jose, California, USA) to infer significant differences between control and treatment groups on biochemical, behaviour and genotoxicity assays. Significantly different treatments were identified using the Holm Sidak or Dunnett's post-hoc test. Analyses were performed with a significance level of 0.05.

3. Results

3.1. Chemical analysis

The quantification of GEM in the exposure solutions showed that nominal concentrations were between 86 and 144% of the measured concentrations (see Table S3). Moreover, HA content did not change the detection of GEM.

3.2. Embryo survival

The survival of zebrafish embryos exposed to GEM, at different HA levels, is depicted in Fig. S1 (Supplementary Material) and the corresponding LC_{10} and LC_{50} values are shown in Table 2. HA levels influenced the toxicity of GEM: at 24, 48 and 72 h, the LC_{50} values for 5 and 20 mg/L of HA were higher than the LC_{50} for the condition of 0 mg/L of HA (Table 2). However, after 96 h of exposure, the LC_{50} values were similar for 0 and 5 mg/L of HA. The generalized likelihood ratio test revealed significant differences in the calculated LC_{50} values between the HA levels of 0 and 20 mg/L for the four days of exposure and between the HA levels of 0 and 5 mg/L in the first 3 days of exposure (Table S4).

3.3. Biochemical assay

The enzymatic activities (ChE, GST, CAT and LDH) of zebrafish embryos exposed to GEM in the presence of different HA levels are depicted in Fig. 1.

Regarding ChE activity (Fig. 1a), increasing HA levels and GEM concentrations led to decreasing ChE activity (GEM: $F = 2.895$, $p = 0.012$; HA: $F = 12.626$, $p < 0.001$). Interaction between the two factors was, however, not observed ($F = 1.182$, $p = 0.305$).

Table 2
Estimated lethal concentrations and slope values (estimate ± standard error) of gemfibrozil (GEM) for zebrafish embryos under different humic acids (HA) levels, after 24, 48, 72 and 96 h of exposure.

Hpf	HA (mg/L)	LC_{10} (mg/L)	LC_{50} (mg/L)	Slope	Model
24	0	10.42 ± 1.97	16.26 ± 1.09	4.23 ± 1.44	W1.4
	5	20.35 ± 12.90	21.96 ± 1.90	28.87 ± 207.95	LL.4
	20	16.54 ± 3.48	21.14 ± 1.00	7.69 ± 5.41	W1.4
48	0	6.52 ± 0.81	12.99 ± 0.59	2.73 ± 0.39	W1.3
	5	18.83 ± 5.01	22.43 ± 0.67	12.56 ± 20.45	LL.3
	20	13.88 ± 1.11	21.02 ± 0.44	4.54 ± 0.77	W1.3
72	0	6.33 ± 0.70	12.62 ± 0.52	2.73 ± 0.33	W1.3
	5	13.97 ± 4.63	19.46 ± 2.05	5.68 ± 4.21	W1.3
	20	13.43 ± 1.89	19.09 ± 0.99	5.36 ± 1.57	W1.3
96	0	5.90 ± 0.71	11.14 ± 0.50	2.96 ± 0.44	W1.3
	5	6.61 ± 1.89	10.87 ± 1.59	4.42 ± 2.01	LL.3
	20	13.29 ± 10.80	14.40 ± 3.04	27.47 ± 206.32	LL.3

Hpf – Hours post-fertilization; HA – Humic acids; LC – Lethal concentration; LL.3 –Log-logistic 3 parameters function; LL.4 –Log-logistic 4 parameters function; W1.3 – Weibul 3 parameters function; W1.4 –Weibul 4 parameters function.

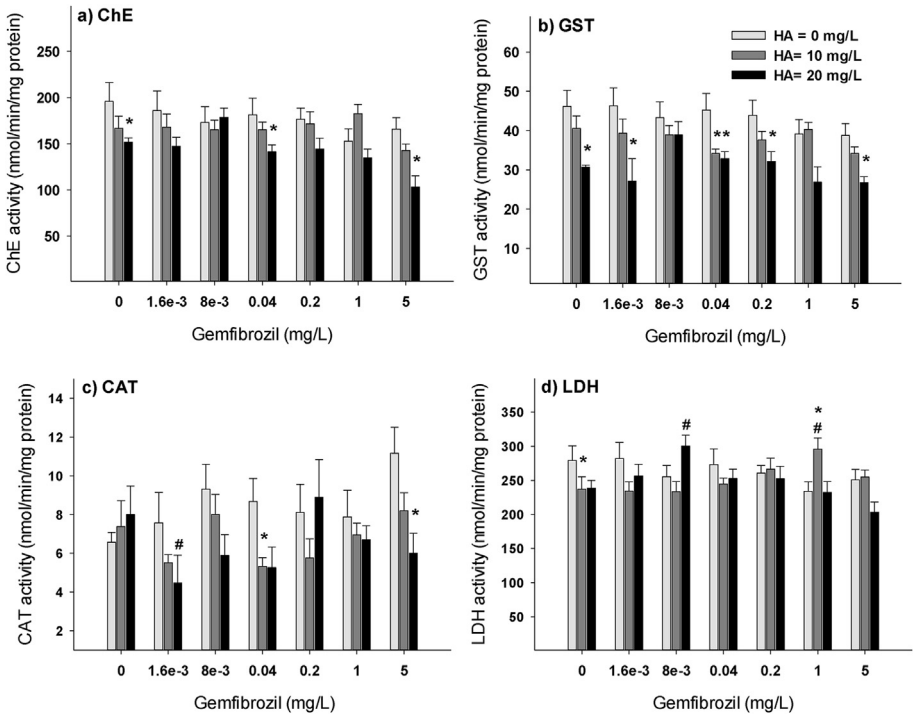

Fig. 1. Enzymatic activities of zebrafish embryos after 96 h exposure to gemfibrozil (GEM), in the presence of different levels of humic acids (HA). Results are presented as mean value ± standard error. a) ChE – Cholinesterase; b) GST – Glutathione *S*-transferase; c) CAT – Catalase; d) LDH – Lactate dehydrogenase. Asterisks (*) indicate significant differences of HA level compared to the respective control within each GEM concentration; cardinals (#) indicate significant differences of the GEM concentration compared to the respective control within each HA level.

GST activity was not affected by GEM exposure ($F = 1.890$, $p = 0.089$, Fig. 1b) but HA levels had a significant effect ($F = 24.653$, $p < 0.001$), showing a clear inhibitory effect at 20 mg/L. No significant interaction between GEM and HA was found ($F = 0.830$, $p = 0.619$).

Concerning CAT (Fig. 1c), both GEM and HA induced significant effects (GEM: $F = 2.976$, $p = 0.010$; HA: $F = 3.523$, $p = 0.033$). High variability of data prevents, however, a clear comprehension of the response patterns. It seems that GEM treatments led to an increase in CAT activity in the absence of HA, although no differences towards control were detected in the multiple comparison test. Increasing HA levels seemed to decrease CAT activity. The two-way ANOVA showed a significant interaction between GEM and HA ($F = 2.468$, $p = 0.007$).

Regarding LDH (Fig. 1d), although no significant GEM ($F = 1.043$, $p = 0.402$) or HA ($F = 1.714$, $p = 0.185$) effects were found, a significant interaction between GEM and HA was observed ($F = 3.201$, $p < 0.001$). No clear pattern of response could be identified for this parameter.

3.4. Behaviour assay

The locomotor activity of zebrafish embryos exposed to GEM at different HA levels is illustrated in Fig. 2. The TD moved by zebrafish larvae was affected by HA and GEM. A lower TD was recorded in larvae exposed to 10 and 20 mg/L of HA when compared to control (HA = 0 mg/L) ($F = 72.315$, $p < 0.001$ for dark period and $F = 5.613$, $p = 0.004$ for light period). In the dark period, GEM led to a concentration dependent inhibition ($F = 3.647$, $p = 0.006$) with differences to control in organisms exposed to 5 mg/L GEM. Although this trend was more pronounced at the highest HA level, the two-way ANOVA did not show a significant interaction between both factors ($F = 1.112$, $p = 0.354$). In the light period, a significant effect of GEM was verified ($F = 3.632$, $p = 0.007$) although no differences towards control were found.

3.5. Comet assay

The DNA damage index of zebrafish embryos exposed to GEM at different HA levels is depicted in Fig. 3. Overall, DNA damage significantly increased with increasing HA levels. A similar profile was displayed in fish exposed to increasing GEM revealing a dose response pattern. In other words, the results show that increasing HA and GEM caused decreasing DNA integrity. Note that in the presence of HA and/or GEM these values are about 5-fold higher than the corresponding value for control conditions (HA = 0 mg/L and GEM = 0 mg/L).

Significant effects were found after individual exposure to HA ($F = 410.403$, $p < 0.001$) and GEM ($F = 553.662$; $p < 0.001$), and a significant interaction between HA and GEM was observed ($F = 158.311$; $p < 0.001$).

4. Discussion

This study aimed to assess the influence of HA on the toxicity of the environmental contaminant GEM to zebrafish. This interference was assessed by studying lethal and sublethal fish responses, at different biological levels (biochemical, cellular and behavioural). HA may affect zebrafish directly (due to HA uptake) and indirectly (by modifying bioavailability of contaminants). Previous studies showed evidences that humic substances can be uptaken (see review by Steinberg et al. (2006)). Upon uptake, HA may contact and penetrate biomembranes potentially inducing adverse physiological and biochemical effects (Steinberg et al., 2006). Indirectly, HA may act by reducing the bioavailability and bioconcentration of contaminants to aquatic organisms, as a result of their high

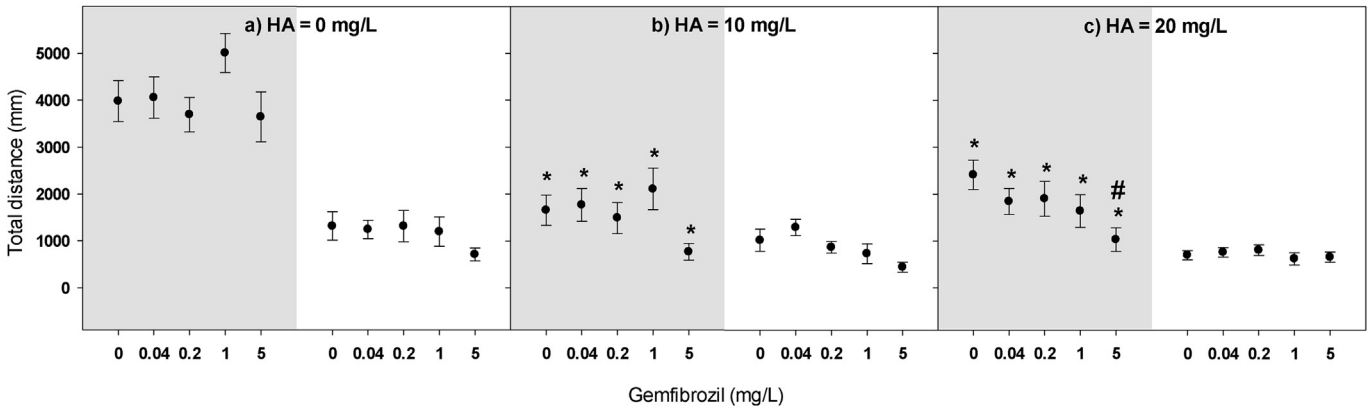

Fig. 2. Total distance moved by zebrafish larvae (120 hpf) after exposure to gemfibrozil (GEM) in the presence of different levels of humic acids (HA), during the light (white area) and dark (grey area) periods monitored. Results are presented as mean value ± standard error. Asterisks (*) indicate significant differences of HA level compared to the respective control within each GEM concentration; cardinals (#) indicate significant differences of the GEM concentration compared to the respective control within each HA level.

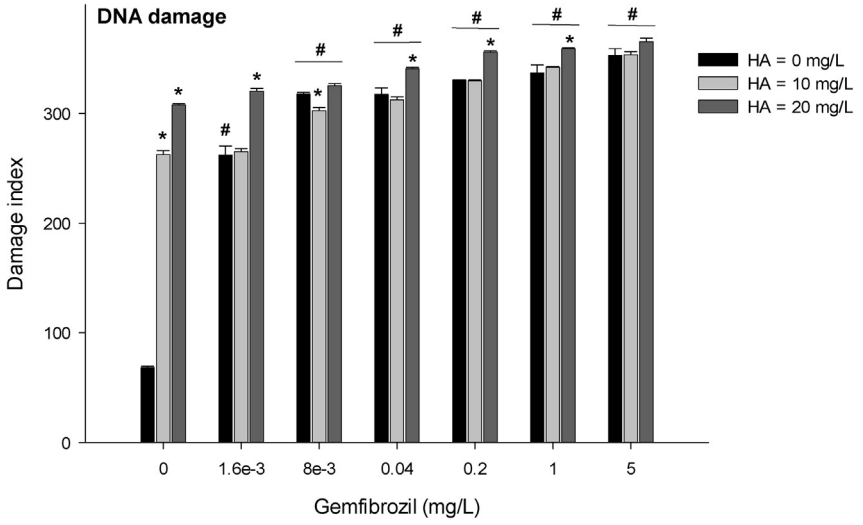

Fig. 3. DNA damage index in zebrafish embryos after 96 h exposure to gemfibrozil (GEM) in the presence of different levels of humic acids (HA). Results are presented as mean value ± standard error. Asterisks (*) indicate significant differences of HA level compared to the respective control within each GEM concentration; cardinals (#) indicate significant differences of the GEM concentration compared to the respective control within each HA level.

complexation ability, although low HA levels have been shown to enhance uptake and bioaccumulation of contaminants (Haitzer et al., 2001).

In this study, HA showed a significant protective role against the lethal effects of GEM, as demonstrated by the increased LC_{50} values in the presence of HA. This protective effect appeared more effective in the first 72 h of exposure, when more pronounced differences between LC_{50} values were observed. This may be related to the complexation of GEM with HA, as reported for other pharmaceuticals (Gu et al., 2007). It is hypothesized that these complexes cannot penetrate the chorion as easily as the components alone, due to their dimensions (Haitzer et al., 1998), consequently reducing GEM bioavailability. In the first 72 h of exposure, the HA level (5 or 20 mg/L) does not seem to influence the magnitude of the protective effect given that the LC_{50} values in the presence of 5 and 20 mg/L HA are similar. After hatching, larvae become more exposed to the toxicant with a consequent increase in toxicity for all HA levels (at 96 h no protective effect of HA was observed). The protective effect of HA against the lethal effects of GEM is in agreement with other studies reporting decreased toxicity of pesticides (Bejarano et al., 2005; Wu et al., 1999), metals (Kamunde and MacPhail, 2011), water disinfectants (Gustavino et al., 2005) and personal care products (Carmosini et al., 2016) in the presence of HA.

Although HA, by themselves, did not affect the survival of zebrafish embryos, they affected most of the tested sublethal endpoints, including biochemical parameters.

In this study, ChE activity was affected by both stressors although the effects of HA were more pronounced (i.e. effects of GEM towards control could not be discriminated). A minor response of ChE activity to GEM exposure is not surprising given that this compound has not been reported as neurotoxic. A previous study reported no effects of GEM (up to 15 mg/L) on *Sparus aurata* brain and muscle ChE activity (Barreto et al., 2018). Similarly, clofibrate (also a fibrate) induced no effect on acetylcholinesterase (AChE) activity in adult mosquitofish (*Gambusia holbrooki*) (Nunes et al., 2004). Few studies have analysed, however, the effects of HA on ChE activity. To our knowledge only Chiang et al. (2016) analysed ChE activity after fish (*Oryzias latipes*) exposure to HA (0.5 mg/L), reporting no effects. In our work, an inhibition caused by HA levels was observed. Such an effect highlights the importance of attending to water characteristics when using biochemical

responses in ecological risk characterization as some parameters such as HA can be confounding factors. Unlike mortality, HA did not seem to change the response of ChE towards GEM, as supported by the lack of significance of the interaction between HA and GEM.

Concerning GST activity, the fact that it was not affected by GEM agrees with a previous study reporting no GEM effects in concentrations up to 15,000 μg/L (Barreto et al., 2018). However, GST was affected by HA, showing a decrease at the highest HA levels, in agreement with works performed with the amphipods *Gmelinoids fasciatus* (Timofeyev and Steinberg, 2006) and *Eulimnogammarus cyaneus* (Timofeyev et al., 2006b). HA may be considered a potent oxidant (Hseu et al., 2014) leading to oxidative stress and reactive oxygen species (ROS) production as reported in studies involving mammal cells (Gau et al., 2001; Hseu et al., 2008). It would be expected that ROS enhancement should trigger alteration in the antioxidant defences, with increases in enzymatic activities including GST. Nevertheless, in the case of ROS overproduction, inactivation of enzymatic defences may occur with damage of cellular macromolecules, such as lipid injury and DNA lesions.

Regarding CAT activity, effects were detected after both GEM and HA exposure although the pattern of response was not clear. An activation of this enzyme was expected based on studies suggesting oxidative stress promotion by HA (Steinberg et al., 2006; Timofeyev et al., 2006a). Concerning GEM, a CAT activity increase was also expected based on a previous study with *Sparus aurata* (Barreto et al., 2018), whereas an inhibition was reported upon exposure to clofibrate in adult mosquitofish (Nunes et al., 2004). The combined effects of both stressors, allied to data variability, likely contributed to the lack of clear response patterns. This suggests that, similarly to what was observed for ChE and GST, HA may be a confounding factor when assessing biochemical effects of pharmaceuticals.

Concerning LDH, an increased activity is commonly interpreted as reflecting a situation of stress/and or exercise, requiring more energy. The exposure to GEM and HA did not produce a clear dose response pattern in LDH activity. Although occasional effects were detected, the lack of a clear trend for this enzyme is unlikely to denote a relevant biological meaning which agrees with a previous study with adult mosquitofish (*Gambusia holbrooki*) exposed to clofibrate (concentrations up to 3 mg/L) (Nunes et al., 2004).

At a behavioural level, the locomotion of zebrafish was affected by both GEM and HA. In general, zebrafish larvae tend to be more active in the darkness (as can be observed in the Fig. 2a), however HA seemed to decrease their activity in dark periods. This might be related to the observed inhibition of ChE by HA, which leads to a disruption in the nervous system function and consequent irregular neuronal and muscular activity (Behra et al., 2002) resulting in locomotor disruption (Kavitha and Venkateswara Rao, 2007; Zhang et al., 2013). The observed effects of GEM on fish behaviour, found in the present study, are in agreement with a previous study that reported significant impairment of zebrafish locomotor activity (as TD) at GEM concentrations equal or above 6 mg/L in dark periods (Henriques et al., 2016). In the dark period, it was evident a dose dependent decrease of TD with increasing GEM concentration, which was more pronounced at the highest HA level. In other words, the observed toxicity was higher in the presence rather than in the absence of HA. This suggests that, unlike for mortality, HA does not play a protective role against GEM toxicity but, instead, contributes to increased toxicity. This result was expected as it followed the toxicity trend observed for both stressors individually.

Considering the genotoxic potential of both stressors, our study showed that both GEM and HA induced DNA damage in zebrafish embryos. Overall, DNA damage index demonstrated a high sensitivity to the tested conditions when compared to the other tested endpoints. DNA damage was found for GEM concentrations equal and above 0.08 mg/L, which is below the environmental concentrations previously reported (Schwab et al., 2005). GEM also induced DNA damage in the aquatic species *Sparus aurata* (Barreto et al., 2017) and *Dreissena polymorpha* (Quinn et al., 2011) after 96 h exposure, which is in agreement with the results of the present study. For HA, a previous study reported their ability to induce DNA damage in human lymphocytes, which was associated with the production of ROS, reactive nitrogen species and increased intracellular Ca^{2+} levels (Hseu et al., 2008). In the present study, as observed for the human lymphocytes, HA demonstrated concentration dependent genotoxicity. Regarding the interaction between GEM and HA, an increased GEM toxicity in the presence of HA was observed, which follows the effects of both stressors individually, suggesting that HA contributes to increased toxicity. A reduction in genotoxicity has been however reported for mitomycin C in the human lymphoblastoid cell line TK6 (Ferrara et al., 2006). The DNA integrity loss found in the present study does not seem related with oxidative DNA damage, based on the results of CAT and GST activities. Nonetheless, further studies should clarify this issue by using repair endonucleases with appropriate specificities (e.g. oxidised pyrimidines, 8-oxo-7,8-dihydroguanine) that can be incorporated into a modified comet assay protocol. It has to be emphasized that the detected damage may be repaired if DNA repair mechanisms are not affected by these compounds.

The hypothesis tested in this study was that increasing DOC concentrations, through HA, would decrease GEM toxicity to zebrafish. This decrease would be a consequence of the high complexation capacity of HA and, thus, reduced bioavailability and bioconcentration of GEM. This was observed for mortality, since increasing HA reduced GEM toxicity. However, the same trend was not observed for sublethal endpoints: the presence of HA elicited *per se* effects on the measured endpoints that, combined with GEM effects, led to increased effects, namely on the locomotor activity and genotoxicity endpoints. This distinct effect may be related with HA properties, being of easy uptake and in contact with biomembranes, triggering several subcellular mechanisms (Steinberg et al., 2008). It can be concluded that, although without lethal toxicity, HA may be considered as a physic chemical/abiotic stressor, adding its effects on several sublethal endpoints to the effects of other contaminants, magnifying the risks for aquatic organisms and populations. This result suggests an increased impact of climate changes to freshwater organisms and justifies further studies on the subject. It is, however, important to note that the results of this study cannot be generalized to the behaviour of DOC, given that in natural waters, HA represent approximately only 4–29% of total DOC (De Schamphelaere and Janssen, 2004; Ma et al., 2001). In addition, available studies demonstrate the importance of the composition/ratio of HA on its effects (Ma et al., 2001). Indeed, the components of humic substances, including HA and fulvic acids, have different affinity for each chemical. For instance, HA have a greater affinity than fulvic acids for binding hydrophobic compounds (De Paolis and Kukkonen, 1997), as is the case of GEM. Thus, the protective role against GEM lethal effects might not be so notorious if natural DOC was used instead of only HA. This is supported by a previous study which reported that HA helped to reduce the negative effects of copper on fish gills better than did natural organic matter (Matsuo et al., 2004). Moreover, the binding capacity of HA may be affected by other environmental factors such as pH, as reported by De Paolis and Kukkonen (De Paolis and Kukkonen, 1997). Finally, the presence of other compounds in the water, such as disinfectants, might also alter HA effects (Gustavino et al., 2005).

The results of the present study emphasize the role that abiotic factors, namely HA, may play on the toxicity of environmental contaminants. The importance of using abiotic factors, such as HA,

as a co-stressor in ecotoxicity tests has been pointed in several studies in the last decades (Burnison et al., 2006; Stuijfzand et al., 1999). Nevertheless, the mechanisms must be investigated for a deeper understanding of HA/DOC effects in a more environmental and realistic perspective. Also, the long-term effects and bioaccumulation of GEM and HA should be investigated, given the slow degradation and high consumption of GEM.

5. Conclusions

HA affected distinctively GEM toxicity to zebrafish embryos. The presence of HA reduced the lethal GEM toxicity to zebrafish embryos especially in the first 72 h of exposure. However, this protective effect was not corroborated at sublethal level as HA *per se* elicited significant adverse effects in some of the tested endpoints. Hence, this study demonstrated that HA may play a determinant role in chemicals toxicity and, thus, it should be considered in ecotoxicity studies aiming at testing realistic scenarios. Moreover, it was also shown that lethality is not enough to characterize combined effects since different patterns of responses may occur at different levels of biological organization. Thus, it is recommended that both lethal and sublethal endpoints are employed to achieve a robust risk assessment.

Acknowledgments

Thanks are due for the financial support to CESAM (UID/AMB/50017 - POCI-01-0145-FEDER-007638), to FCT/MCTES through national funds (PIDDAC), and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020; Portuguese Foundation for Science and Technology (FCT) within project CLIMATOX (PTDC/AAGGLO/4059/2012); Post-Doc grant SFRH/BPD/90521/2012 attributed to Inês Domingues; Miguel Oliveira had financial support of the program Investigador FCT (IF/00335-2015), co-funded by the Human Potential Operational Programme and European Social Funds. Olga Koba and Pham Thai Giang were supported by the Ministry of Education, Youth and Sports Czech Republic with projects CENAKVA (No. CZ.1.05/2.1.00/01.0024) and CENAKVA II (No. LO1205 under the NPU I program) and by the Grant Agency of University of South Bohemia (USB) (No. 012/2016/Z).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2018.12.133.

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